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Pre- and Postfusion Regulation of Transmitter Release

Review

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Introduction

Synaptic transmission is a highly regulated function of the nervous system. It is performed by three sets of processes: presynaptic processes that start with the invasion of the action potential into the presynaptic nerve terminal and end with the release of transmitter from synaptic vesicles; processes in the synaptic gap; and postsynaptic processes. This article deals mainly with one specific aspect of the presynaptic process of transmitter release: the events that occur after the fusion of the synaptic vesicle with the presynaptic surface membrane. We will attempt to examine the possibility of a combined pre- and postfusion regulation of transmitter release.

The most prevalent view regarding transmitter release from presynaptic nerve terminals states that the regulation is exerted at the steps preceding the fusion of the synaptic vesicle with the surface membrane. Recent results from our two laboratories, together with observations made by others, raise the possibility that regulation of transmitter release continues also after the fusion process.

The idea that transmitter release is regulated after fusion of the synaptic vesicle is based on a number of experimental observations, done out of necessity, on different preparations. We would like to describe these observations, then present the hypothesis, and finally discuss their possible implications and limitations. Since the postfusion hypothesis is a direct consequence of the vesicular hypothesis for transmitter release, we would like to summarize it first.

The Vesicular Hypothesis of Neurotransmitter Release

There are two distinct pathways of transmitter release in the nervous system: quantal release (Katz, 1969) and molecular leakage (see Fletcher and Forrester, 1975; Kuffler and Yoshikami, 1975; Katz and Miledi, 1977; Schwartz, 1987). In this article, we deal only with quantal transmitter release, which is the predominant secretion pathway in the nervous system and in many other secretory cells.

The fact that transmitter is released from nerve terminals as multimolecular quantal packages was first shown at the neuromuscular junction by Bernard Katz and his colleagues (Fatt and Katz, 1952; del Castillo and Katz, 1954; Katz, 1993) and thereafter in many other synapses (Korn and Faber, 1991; Stevens, 1993; Hessler et al., 1993; Jonas et al., 1993; Bekkers, 1994; Von Kitzing et al., 1994; Edwards, 1995; Bekkers and Stevens, 1995). After the discovery that nerve terminals contain vesicles (Palade, 1954; Robertson, 1956), it was proposed that the synaptic vesicles are the structural basis for quantal liberation of transmitter (del Castillo and Katz, 1957; Katz, 1969).

The vesicular hypothesis of transmitter release states that the fusion of a vesicle with the surface membrane and the exocytotic discharge of transmitter stored inside the vesicle generate these quanta. The recent development of high resolution techniques to monitor exocytosis have definitively confirmed the vesicular hypothesis of release.

The initial examination of the idea that the fusion of synaptic vesicles with the surface membrane should increase its area and thus its capacitance was done already in 1979 (Gillespie, 1979). The patch–clamp technique (Hamill et al., 1981) allowed the introduction (Neher and Marty, 1982) of a way to monitor the fusion of individual secretory vesicles with the plasma membrane. The technique consists of measuring the cell membrane capacitance, which is directly proportional to the cell membrane surface area. Upon fusion of a secretory vesicle, the cell membrane area increases stepwise by an amount equal to the granule membrane area (Fernandez et al., 1984; Lindau and Neher, 1988). Wightman et al. (1991) introduced high resolution amperometry as a way to monitor exocytotic release. They monitored the release of secretory products by measuring the oxidation of electroactive substances (i.e., serotonin, catecholamines, dopamine, etc.) with a carbon–fiber microelectrode placed near a cell. The combination of patch–clamp and amperometric techniques demonstrated that upon the fusion of a secretory vesicle (detected by the capacitance measurements), a spike of release of secretory products was seen, directly confirming the vesicular hypothesis of release in at least two cell types: mast and chromaffin cells (Alvarez de Toledo et al., 1993; Robinson et al., 1995; Oberhauser et al., 1996).

Regulated and Constitutive Vesicular Release

Vesicular release can be subdivided into two broad categories: regulated release, where the exocytotic release is the direct response to a stimulus and a constitutive release (see Ma et al., 1995). In this article, we deal with regulated release of transmitter only and ask where the regulation takes place. There are a wide variety of experimental lines of evidence that a very substantial regulation takes place before the fusion of the vesicle with the synaptic membrane.

The regulated release of transmitter occurs most probably at the active zone (Heuser et al., 1974; Ceccarelli et al., 1979; Heuser and Reese, 1981), where the vesicles and the molecular machinery for release are located (Robataille et al., 1990; DeBello et al., 1993; Südhof, 1995; Schweizer et al., 1995). In the original hypothesis of vesicular exocytosis, the mechanism of

release from the fused vesicle was not specified. It was assumed over the years (but never tested) that the release of neurotransmitters occurred after vesicular fusion as a rapid and catastrophic "all or none event." Not surprisingly then, the majority of the studies attempting to understand regulated secretion have focused on prefusion-triggering mechanisms such as intracellular calcium homeostasis, the regulation of ion channels in the surface membrane of the secretory cells, and other forms of intracellular signaling (see Rahamimoff, 1979; Rahamimoff et al., 1980; 1989; Gomperts, 1990). Alternatively, there have been attempts to identify the effector proteins (see Volknandt, 1995).

The typical trigger for regulated transmitter release is the local increase in the intracellular calcium concentration ($[Ca]_{in}$) (Llinas et al., 1982; 1992; Chad and Eckert, 1984; Fogelson and Zucker, 1985; Fidler Lim et al., 1990; Lindau et al., 1992; von Ruden and Neher, 1993; Parsons et al., 1994; Mintz et al., 1995; Matthews, 1997). Hence, factors that affect the activity of the calcium channels in the nerve terminals affect the regulated release of transmitter. Such factors include hormones and other transmitters that act on the activity of the calcium channels, usually via G proteins (Hille, 1994b; Dolphin, 1995), or factors that act on potassium channels and change the duration of the action potential (Klein and Kandel, 1978; 1980; Klein et al., 1980; Shapiro et al., 1980; Eliot et al., 1993). All of these and many other determinants act on the regulation of the rate of fusion of the vesicles with the surface membrane and constitute part of the prefusion control of transmitter release.

How Is Transmitter Released from Vesicles?

After fusion of a synaptic vesicle, the release of secretory products has been assumed to represent the unregulated 'dumping' of the mixture of substances stored in the vesicle. It is generally assumed that the secretory products are stored inside the secretory vesicles as a solution or as simple aggregates or precipitates (Gerdes et al., 1989). The latter view further proposes that upon exocytotic fusion, this precipitate disassembles, allowing the release of secretory products through the exocytotic fusion pore by simple unregulated diffusion (Bruns and Jahn, 1995).

The recent development of techniques to probe exocytotic release at the level of single vesicles has permitted a critical examination of this untested but well-accepted hypothesis. These studies showed that release has a complex time course. In particular, the bulk of release is typically delayed with respect to vesicle fusion (Chow et al., 1992; Alvarez de Toledo et al., 1993; Wightman et al., unpublished data). This delay between the fusion of the vesicle (measured by capacitance) and the appearance of the transmitter in the extracellular space (measured by amperometry) casts a doubt on the simplistic view of an 'all or none' unregulated release of the content of the vesicle. This doubt is strengthened by a simple calculation of the rate and amount of release of transmitter through an open fusion pore (vide infra): the rate of release was shown to be at least 50 times less than that predicted by simple diffusion (Alvarez de Toledo et al., 1993). These observations suggested that exocytotic release may be regulated after the exocytotic fusion pore opens (Neher, 1993; Alvarez de Toledo et al., 1993; Wightman et al., unpublished data). We would like first to estimate this discrepancy from the known properties of the exocytotic fusion pore and then to propose that they originate (at least in part) from the supply of ions to the interior of the vesicle, which contains an ion exchange matrix (Nanavati and Fernandez, 1993; Monck and Fernandez, 1994; Yakir and Rahamimoff, 1995).

The matrix of the secretory granules found in mast cells is made of heparin proteoglycans, which are densely charged and fill the volume of a secretory granule. Uvnäs and collaborators have demonstrated that isolated matrices of mast cell granules are cation exchangers that can store large amounts of histamine, serotonin, and other cations (Uvnäs and Åborg, 1983; 1989). In this review article, we would like to examine the possibility that some of the conclusions reached for mast cells are applicable to the exocytotic release from nerve terminals.

The Exocytotic Fusion Pore

The fusion pore was first observed in degranulating mast cells using a combination of rapid-freezing techniques and freeze-fracture electron microscopy (Chandler and Heuser, 1980). The development of patch-clamp-capacitance techniques permitted, for the first time, direct observation of the activity and size of single fusion pores in isolated cells undergoing exocytosis (Neher and Marty, 1982; Fernandez et al., 1984; Zimmerberg et al., 1987; Breckenridge and Almers, 1987a). The patch clamp has a high resolution, allowing a characterization of the fusion pore in terms comparable to that of single ion channels. These measurements revealed that the exocytotic fusion pore opens abruptly and is initially small (diameter: 1–2 nm). After opening, the pore either closes again completely (transient fusion, sometimes called "flicker") or expands irreversibly (irreversible fusion).

Amperometry measures the current produced when molecules are oxidized by the exposed surface of a carbon–fiber microelectrode. This technique has an extraordinary sensitivity and time resolution and easily captures the release of secretory products during single exocytotic events. Amperometric measurements in chromaffin and mast cells have shown that a single fusion event results in a spike-like amperometric current (Leszczyszyn et al., 1990). A detailed analysis of catecholamine release indicated that the shape of the spikes could not be explained purely by diffusion from a point of instantaneous release (Schroeder et al., 1992; Jankowski et al., 1993; Wightman et al., unpublished data). Furthermore, the spikes of catecholamine release were frequently shown to be preceded by a slower phase of release that was called the "foot" of the spike (Chow et al., 1992). These observations led to the proposal that catecholamine release was finely regulated by fluctuations in the size of the fusion pore that links the lumen of the secretory vesicle with the extracellular environment during exocytosis (Chow et al., 1992; Neher, 1993; Jankowski et al., 1993; Wightman et al., unpublished data).

Neurotransmitter Release through a Fusion Pore

Fusion pores can be described as ionic pores. The elementary properties of ionic pores have been extensively discussed (Hille, 1994a; Sakmann and Neher, 1995). It is possible to apply Ohm's law and the diffusion equation to obtain a simple description of the basic properties of such pores. The electrical resistance of a hypothetical fusion pore is the sum of the resistance of a cylinder with a radius r and length 1 and the access resistances into the internal and external mouths of the pore.

$$R = 2 \cdot R_{mouth} + R_{pore} = \frac{\rho}{\pi r^2}\left(1 + \frac{\pi r}{2}\right)$$

where ρ is the resistivity of the electrolytic solution in the pore and surrounding solutions (about 100 Ohm $\times$ cm). We take an arbitrary fusion pore with a radius of 1.1 nm and a length of $\sim$10 nm. Such a pore will have a total resistance of $\sim$3 Gohm, corresponding to a pore conductance of $\sim$300 pS; this value is consistent with measurements of the early fusion pores in mast cells (Spruce et al., 1990).

The size of a fusion pore will determine its ability to allow the diffusional passage of secretory products down their concentration gradient and into the extracellular space. We can calculate the efflux of secretory products through a fusion pore (ϕ) from the flow of serotonin into the pore's mouth and the flow through the pore proper (Hille, 1994a).

$$\phi = \frac{\pi r^2 D c}{1 + \frac{\pi r}{2}}$$

As an example, we can calculate the efflux of serotonin through the fusion pore of a mast cell granule. If we assume that the diffusion coefficient is $D \sim 10^{-5}$ cm^2/s and that the granule contains c = 200 mM, then a fusion pore with a radius of 1.1 nm will permit an efflux of 3.9 $\times$ 10^7 molecule/s. Let us compare this number with the number of molecules available at the internal mouth of the pore. The rate at which molecules enter the mouth of such a fusion pore can be found (by first approximation) by solving the diffusion equation assuming that the molecules come from an infinite medium of concentration c and hit a disk of radius r that acts as a sink (Hille, 1994a). Under these conditions, the flow of molecules to the mouth of the pore is given by:

$$\phi_{mouth} = 2\pi r D c$$

For a disk of radius 1.1 nm, we calculate that 8.3 $\times$ 10^8 serotonin molecule/s will become available at the mouth of such a pore. Clearly, for a simple diffusion from a medium with $D \sim 10^{-5}$cm^2/s, the flow via the fusion pore is not limited by the diffusion of molecules into the pore's mouth. This is about 20 times more that the pore can pass. Thus, if these assumptions are valid, the size of a cylindrical fusion pore (r = 1.1 nm; 1 = 10 nm; $D \sim 10^{-5}$ cm^2/s) should limit the magnitude of the release of serotonin. We shall see that for mast cells, the actual release of serotonin is substantially smaller than that predicted by these simple considerations. To understand the origin of this discrepancy, it is imperative that we examine how secretory products are stored before and mobilized after the exocytotic fusion of a vesicle.

The Contents of Secretory Granules and of Synaptic Vesicles

In cholinergic synaptic vesicles, one finds that the lumen of the vesicles contains a grossly hyperosmotic concentration of secretory products; the same applies to the large mast cell granule. Mast cell granules contain at least 400 mM histamine, 200 mM serotonin plus ATP, Ca^{2+} (Nicaise et al., 1992), and large amounts of a heparin proteoglycan (Rabenstein, 1987). The small synaptic vesicles of *Torpedo* contain about 0.8 M acetylcholine, plus high concentrations of Ca^{2+}, magnesium, and a variety of nucleotides (such as ATP and GTP) as well as other components (Parsons et al., 1993; Zimmermann et al., 1993). It is difficult at present to construct a complete 'balance sheet' of the cationic and anionic charges in the synaptic vesicle, but there is no doubt that the number of soluble cations exceeds by far the number of soluble anions.

It has long been assumed that the secretory products are in solution 'waiting' to be released. For example, the clear, electron-lucent appearance of cholinergic vesicles suggests that their contents is indeed in solution and that the entire cocktail of soluble constituents is released on exocytosis (Zimmermann et al., 1993). However, storing a hyperosmotic mixture of secretory products in solution poses problems that have long puzzled investigators. An energy-consuming and effective uptake mechanism that stores the secretory products into a complex precipitate has been proposed (Gerdes et al., 1989). However, the dissolution of a storage precipitate could be prohibitively slow. It seems also unlikely that the secretory products are simply kept in solution, generating large osmotic gradients and requiring a continued energy consumption.

The Secretory Granule Matrix: An Ion Exchange Gel

For a number of years, Uvnäs and Åborg (1989) and Verdugo (1991) have proposed two simple solutions for the storage of transmitters in secretory granules and vesicles. Uvnäs and Åborg proposed that the matrix of a secretory granule functions as an ion exchange resin, where the storage and release of ionic secretory products occurs by stochiometric exchange of secretory products by counterions. Ion exchange resins are built as insoluble cross-linked polymers with a high density of fixed charges. Since electroneutrality must be satisfied at all times, an equal number of counterions must neutralize the fixed charges in the resin. Hence, large amounts of charged secretory products could be stored as counterions, and then rapidly mobilized for release by ion exchange (Uvnäs and Åborg, 1989). The matrix of a mast cell granule is made of a densely charged, anionic, heparin proteoglycan hydrogel. Uvnäs and Åborg demonstrated that the isolated mast cell granule matrices had the properties of ion exchange resins. Ion exchange provides a simple mechanism for the storage and release of large amounts of counterions. However, the role of ion exchange in the release of secretory

products in vivo remained untested until recently (Marszalek et al., 1996).

By studying the properties of mucin granules, Verdugo (1991) proposed another solution for the storage of secretory products. He showed that the polycationic mucin core of goblet cell granules had the properties of "smart" polymeric hydrogels (1991). A "smart" hydrogel is so called because of its ability to respond to small environmental changes (ions, electric fields, light, temperature, etc.), with large volumetric changes caused by a phase transition mechanism (Tanaka, 1981). (Smart hydrogels are used in the design of novel sensors and electromechanical devices [Tanaka et al., 1982]). Verdugo proposed that a condensed, "smart" hydrogel could trap secretory products, as in a molecular cage. Furthermore, since a collapsed hydrogel behaves as a single particle, it practically does not create an osmotic gradient. Then, upon triggering a phase transition, an explosive decondensation would release the secretory products as in a "jack-in-the-box" (Verdugo, 1991). The ion exchange "smart" gels provide a simple mechanism for electrostatically storing large amounts of charged molecules without creating osmotic gradients.

It is likely, therefore, that both mechanisms, ion exchange and "jack-in-the-box", contribute to the release of the large variety of secretory products that are known to be stored in vesicles.

The Diffusion of a Neurotransmitter within Its Storage Matrix Regulates Release during Transient Fusion

The mechanisms involved in the electrostatic storage of a neurotransmitter within a gel matrix are likely to regulate its release. This form of regulation is particularly relevant during transient fusion events (Alvarez de Toledo et al., 1993). A release mechanism that relies on transient fusion would, at first glance, be irrelevant. For example, a typical small synaptic vesicle with a radius of 25 nm, filled with a transmitter, will empty through a 300 pS fusion pore in about 220 μs (Almers et al., 1989). Hence, a transient fusion event that lasts longer than 220 μs would allow the entire vesicular contents to be released. Transient fusion events would have to last $<$220 μs to become relevant. This calculation is based on the assumption that the diffusivity of the neurotransmitters in the interior of the vesicle is similar to its diffusion in water ($\sim 10^{-5}$cm^2/s) (Almers et al., 1989).

Recently, Marszalek et al. (1996) demonstrated that the release of serotonin from isolated secretory granules is limited by the diffusivity of serotonin within the gel matrix that fills the lumen of the secretory granule. They estimated a diffusion coefficient for serotonin, within the matrix, of 1.3×10^{-8}cm^2/s, which is almost three orders of magnitude smaller than that in the bulk ($\sim 10^{-5}$cm^2/s) but reasonable for a charged molecule diffusing within an ion exchange gel (Helfferich, 1962). Given that serotonin needs to diffuse within the granule matrix to reach the internal mouth of the fusion pore, Marszalek et al. (1996) calculated that the number of serotonin molecules reaching the mouth of a fusion pore with a radius of 1.1 nm is only $\sim 1 \times 10^6$ molecule/s. By contrast, if the remainder of the pore is water filled and has a diffusion coefficient equal to that in the bulk, they calculated that the pore will sustain a flow of about 4.0×10^7 molecule/s (see above). Thus, the number of molecules reaching the mouth of the pore is calculated to be only $\sim$1/40 of the efflux that the pore can sustain. Thus, serotonin release through a small fusion pore is limited by the diffusion of serotonin to the mouth of the pore rather than by the pore. These calculations were found to agree well with the experimental findings (Alvarez de Toledo et al., 1993).

It is likely that the diffusivity of a neurotransmitter in the lumen of a small synaptic vesicle is also limited by an ion exchange gel matrix. If we assume that the diffusivity of a transmitter is 10^{-8}cm^2/s, as it is in the mast cell, it will take approximately 10 ms for a small synaptic vesicle ($r = 25$ nm) to empty through a fusion pore of 300 pS. Under these conditions, transient fusion pore openings that lasted 0.5–1 ms would release only a fraction of the vesicular contents. It is possible that such transient openings release what is commonly understood as a "quantum." If this speculation is correct, then a substantial part of the coefficient of variation of the unitary quanta may be attributed to the duration of the opening of the fusion pore.

It is important to note that a low neurotransmitter diffusivity within the gel matrix of a secretory vesicle will only limit the amount of neurotransmitter molecules reaching the mouth of the fusion pore (Marszalek et al., 1996). However, once a neurotransmitter molecule reaches the mouth of the fusion pore, it will then enter the pore and diffuse into the synaptic cleft, and it will no longer be affected by the ion exchange gel. Thus, the synaptic delay should not be affected by a mechanism of release that is regulated by an ion exchange gel matrix; only the number of neurotransmitter molecules being released in a given time are affected. As these arguments indicate, the diffusivity of a neurotransmitter within the lumen of the secretory vesicle may be another important parameter in defining the size of the "quantum."

Are There Ion Exchange Gels in Synaptic Vesicles?

Since the molecular mechanisms of fusion are thought to be highly conserved, we would like to think that the mechanisms regulating the postfusion release of secretory products are not limited to mast cells but are widespread. In particular, we think that similar ion exchange gels regulate the storage and the postfusion release of neurotransmitters. This view predicts that synaptic vesicles contain ion exchange gels for the storage and the postfusion regulation of the release of secretory products.

The 'clear' appearance of the synaptic vesicles in the electron microscope seems to be misleading. EM studies require dehydration and fixation of the vesicles, thus destroying the properties of a functional ion exchange gel. Furthermore, some gels, like those made of sugars, cannot be made electron dense and therefore would not be seen, resulting in the 'clear' appearance of many synaptic vesicles.

Like mast cells, secretory granules from many cells, including clear synaptic vesicles, contain charged proteoglycans, suggesting that the mechanisms described

in the previous sections are widespread (Stadler and Dowe, 1982; Carlson and Kelly, 1983; Uvnäs and Åborg, 1983; Wagner, 1985; Walker et al., 1986; Kiene and Stadler, 1987; Stadler and Kiene, 1987; Kuhn et al., 1988; Uvnäs and Åborg, 1989; Volknandt, 1995). For example, the ubiquitous synaptic vesicle protein SV2 is a keratan sulfate proteoglycan (Feany et al., 1992; Scranton et al., 1993). It is possible that these proteoglycans form a charged gel matrix with the properties of an ion exchanger, similar to those found in the mast cell.

The interior of the synaptic vesicles of the *Torpedo* electromotor nerves contains about 0.8 M of acetylcholine and about 0.12 M of ATP, which is also negatively charged. Thus, the positive charge of the transmitter molecules of acetylcholine is partially neutralized by the soluble ATP and partially by the negatively charged polymer matrix. We do not have a complete stochiometric picture of the distribution of the acetylcholine molecules among the various anions inside the synaptic vesicle. It is clear, however, that part of the acetylcholine is neutralized by mobile charges and part by the fixed charges of the ion exchange matrix. Thus, two of the important components of the postfusion hypothesis are present not only in mast cells but also in synaptic vesicles of Torpedo. Synaptic vesicles contain an ion exchange matrix, and their content is known to be hyperosmotic.

Is the Quantal Size Affected by the Fusion Pore Properties in Synapses?

Due to their small size and fragility, synapses remain inaccessible to some of the most powerful techniques for examining the mechanisms of release. Despite these limitations, it may be possible to create a more detailed picture of the synaptic events by using a wide variety of biochemical and electrophysiological observations. These observations have been combined into quantitative models that predict the time course and size of a synaptic response (Wathey et al., 1979; Bartol et al., 1991; Clements et al., 1992). All of the models have assumed that vesicular fusion is an all or none event and that upon fusion, release of the entire contents of the vesicle (a quantum) is instantaneous. However, Van der Kloot (1995) noted that the rise times of miniature end plate currents in the frog neuromuscular junction are considerably longer than those predicted by the models (see also Cherki-Vakil et al., 1995). Van de Kloot (1995) proposed that the assumption of instantaneous release is probably incorrect and that acetylcholine is released over a period, after vesicle fusion.

It is well known that release of transmitter undergoes quantal variations. But in addition to the quantal variations, there are a number of instances where other sources of variance have been detected. Among them are the "subminis" that have been nicknamed "dwarfs" by Bernard Katz. The amplitude of these events is substantially smaller than that of the typical quantal event (Kriebel and Gross, 1974; Wernig and Stirner, 1977). Several suggestions were put forward to explain the origin of the dwarfs: the release of a partially filled vesicle, the release of a subquantum, and the release of the content of a vesicle away from the active zone. Another interesting observation is that the size of the "quanta," as measured from the miniature end plate potentials, can be altered several fold by a variety of experimental conditions (Van der Kloot, 1991). These observations were interpreted as an indication that the quantity of neurotransmitter stored into a vesicle can be changed (Van der Kloot, 1991). However, an additional interpretation is feasible: the amount of neurotransmitter released during a fusion event may be less than the full vesicular contents. In the synaptic vesicles of the *Torpedo* electromotor neurons, the acetylcholine content is several orders of magnitude larger than the amount required to produce a quantum (Parsons et al., 1993). When a small, diffusion-limited fusion pore regulates the amount of neurotransmitter released during a transient fusion event, the duration of the transient fusion would be one of the determinants of the size of the quantum. It is possible that under certain experimental conditions, the duration of transient fusion events is reduced, thus decreasing the size of the postsynaptic response, leading to the appearance of the "dwarfs."

Transient Vesicle Fusion and Exocytotic Release

Although not anticipated in the original quantal hypothesis, transient vesicular fusion and partial release of vesicular contents are now well established experimental observations (Fernandez et al., 1984; Spruce et al., 1990; Alvarez de Toledo et al., 1993). The percentage of transient fusion events in cells having small vesicles (i.e., neurons; 50–200 nm) is, at present, not known because most events are below the resolution of patch–clamp capacitance measurements. However, it is likely that transient fusion is a significant form of neurotransmitter release. Early studies estimating vesicle recycling during nerve stimulation showed that the decrease in the number of vesicles associated with exocytotic release seemed to be smaller than the estimated number of transmitter quanta liberated (Ceccarelli et al., 1972; 1973; Heuser and Reese, 1973; Heuser et al., 1974; 1979; 1981; Landis et al., 1988; Heuser, 1989; Valtorta et al., 1990).

More recently, Betz and coworkers developed an optical method to estimate vesicle traffic in the nerve terminal (Betz and Bewick, 1992a; Betz et al., 1992a; 1992b; Betz and Henkel, 1994; Betz and Wu, 1995). They used the fluorescent dye FM1–43 to stain biological membranes. During the staining period, the dye is taken up by the surface membrane of the nerve terminal. When the vesicles are recycled, the surface membrane is internalized, and the dye is a part of the vesicle membrane. After the removal of the dye from the external solution, a complete fusion of a stained vesicle with the surface membrane will cause a destaining. They were able to compare the rate of destaining, which most probably represents a complete fusion of the vesicle with the surface membrane of the presynaptic terminal, with the rate of actual transmitter release (Betz and Bewick, 1992b). Their data shows clearly that under certain experimental conditions, transmitter release exceeds by far the rate of destaining. Recently, Betz and collaborators also reported that a protein kinase inhibitor, staurosporine, blocked evoked destaining of FM1–43 from frog

motor nerve terminals (Betz and Henkel, 1994). Surprisingly, staurosporine did not affect synaptic transmission. A possible interpretation of these results is that in the presence of staurosporine, most synaptic transmission events occur by transient fusion, leaving the dye entrapped in the vesicle while allowing the release of acetylcholine. It has been shown that, during transient fusion events, tension in the granule membrane drives a large flux of lipids through the fusion pore, from the plasma membrane into the granule membrane (Monck et al., 1990). If similar events take place during the transient fusion of a synaptic vesicle, it is easy to see how the fusion pore will act as a valve, letting in the FM1–43 dye but not allowing it to diffuse out.

(For other lines of evidence suggesting the existence of transient fusion in the process of transmitter release, see Fesce et al., 1994).

The Role of Counterions in Release

The results of the release studies suggest that ion exchange mechanisms are important for the release of charged secretory products. They raise a fundamental question: what is the source of the counterions that are required for release? In an intact cell undergoing exocytosis, the fixed charges of an ion exchange gel must always be compensated by counterions of the same charge as the charged secretory products. The release of these products requires a stochiometric exchange by another counterion. For a positively charged neurotransmitter such as acetylcholine, there would be a strict requirement for cations during the actual release process. The same requirement applies to mast cells. In the case of the mast cells, the granule matrix is made of a heparin proteoglycan gel with a high density of negative charges. The fixed charges are compensated by an equal number of cationic secretory products (e.g., histamine, serotonin, and Ca^{2+}). Thus, the release of a positively charged substance, such as acetylcholine or serotonin, can occur in one of two possible ways: the transmitter can be accompanied by one of the soluble anions that exist inside the vesicle, or the transmitter can be exchanged by cations from the extravesicular environment. We do not know at present the contribution of these two potential mechanisms to release, since we do not have a complete 'balance sheet' of the soluble ions or knowledge of the relative binding constants. However, the large number of negative charges in the intravesicular gel makes us assume that the second mechanism is probably predominant.

If there is a strict requirement for counterions in these synapses and secretory cells that release positively charged transmitter, then one may speculate that in central synapses where negatively charged transmitter is liberated, the ion exchange gel will be positively charged, and release will have a similar requirement for anions.

Counterion Sources: Ion Channels and Other Transporters in the Synaptic Vesicle Membrane

Figure 1 shows some simple considerations of ionic exchange and its possible regulatory mechanisms. To illustrate the problem, let us use as an example the liberation of serotonin from mast cells, although similar processes probably occur in the release of acetylcholine from cholinergic nerve terminals. Release of an existing cation from the vesicle can occur only by exchange with a new cation (Figure 1A). Otherwise, the release will be impeded by a diffusion potential. Thus, release must be regulated by the availability of new cations for ion exchange and by their ability to move within the matrix. During release, the serotonin stored in mast cell granules is released through the fusion pore while the negative charges of the sulfate groups of the heparin proteoglycan matrix remain fixed inside the granule. Since electroneutrality must be obeyed, the serotonin molecules cannot simply escape, but they must be stoichiometrically exchanged by an equivalent number of charges. Thus, the release of serotonin requires the entry into the granule of counterions that will replace the lost charges. In an intact secretory cell, the intracellular electrolytes bathing the vesicle membrane and the extracellular electrolytes are different. Both sources may supply counterions to displace secretory products from the granule matrix and through the fusion pore. In the case of an intracellular source of counterions, their flow through the vesicle membrane may be regulated by the activity of vesicle membrane proteins, such as ion channels or electrogenic ATPases. In contrast, an extracellular source of counterions will only depend on an open fusion pore.

There are several reports of ion channels found in the membrane of secretory vesicles (Rahamimoff et al., 1988; 1989; Arispe et al., 1992; Hirashima and Kirino, 1992). A flow of cations through an open ion channel in the granule membrane would force an ionic current through the ion exchange gel. The incoming cations may displace the stored secretory products by ion exchange and force them through the fusion pore. Furthermore, a channel-driven ion exchange should be voltage dependent because the total cationic current will depend on the driving force.

Alternatively, electrogenic ATPases could be an important source of counterions. For example, the vacuolar type of the H^+ATPase acidifies the lumen of secretory vesicles. An ouabain-sensitive Na^+/K^+ ATPase has been found to regulate the pH of endosomes (Fuchs et al., 1989). Ca^{2+} ATPases have been found in secretory granule membranes (Nicaise et al., 1992).

Most synaptic vesicles and secretory granules are too small for a direct measurement of ion channel activity by the patch–clamp technique. The size of synaptic vesicles is much smaller than the opening of the patch pipette. Hence, a fusion method was introduced, which allows the fusion of a large number of synaptic vesicles into a giant structure: the giant vesicle (Rahamimoff et al., 1988). This giant vesicle is suitable for patch recording, in the 'vesicle-attached' and in the excised-patch configurations of the patch–clamp technique. Among the different channels found in the vesicle membrane, the most abundant is the nonspecific ion channel (Yakir and Rahamimoff, 1995). It has three properties that may be very relevant to the postfusion hypothesis presented in this article. First, it is nonspecific in its permeability properties. It allows the passage of cations

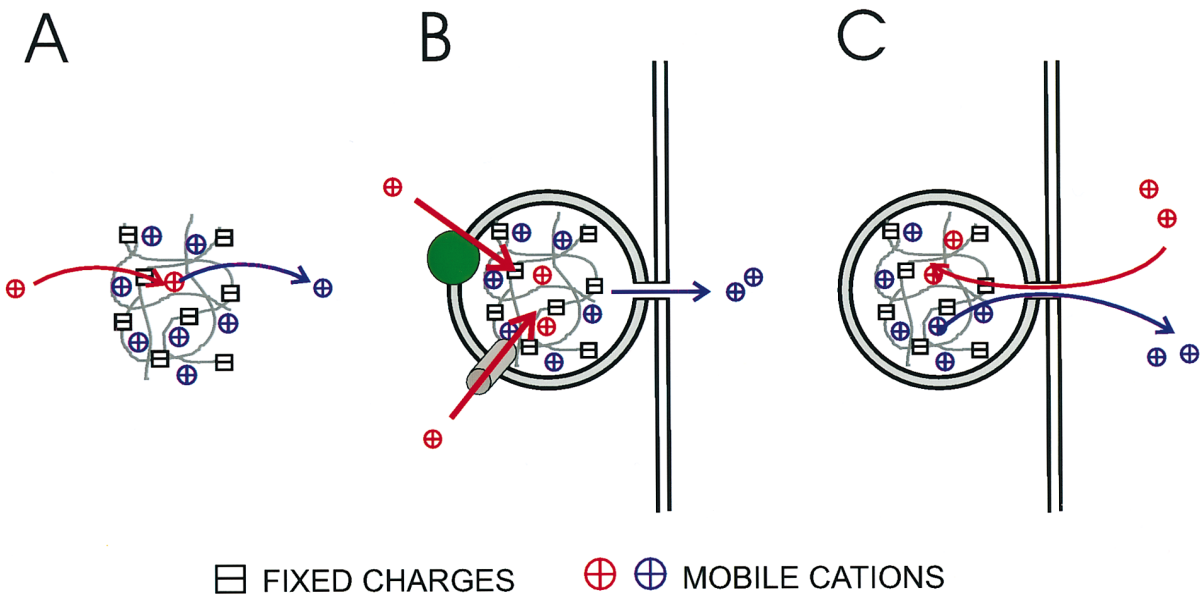

Figure 1. Hypothetical Mechanisms for an Ion Exchange–Dependent Secretory Response

(A) The fixed negative charges of an ion exchange gel are exactly compensated by an equal number of cationic secretory products. Release by ion exchange consists in replacing an existing cation (blue) by a new cation (red). The movement of all ions through the hydrogel is determined by its ion exchange properties. The opening of a fusion pore triggers ion exchange from two sources: (B) through the granule membrane and (C) through the fusion pore. In both cases, the displaced secretory products exit through the fusion pore.

(B) The flow of counterions through the granule membrane is regulated by electrogenic pumps (green circle) or by ion channels (gray cylinder).

(C) The flow of counterions through the fusion pore is regulated by its size and the properties of the fusion pore interface.

and anions according to their electrochemical gradient. Second, it is large and has a conductance of several hundred picosiemens. Third, it is highly voltage dependent. Under normal circumstances, the synaptic vesicle membrane (Michaelson and Angel, 1980; Angel and Michaelson, 1981) and the membranes of other secretory vesicles are electrically charged (~+70 mV) (Breckenridge and Almers, 1987b). Since the nonspecific ion channel is highly voltage dependent, it is fully activated when the vesicle membrane is depolarized near zero mV and is deactivated when the membrane is polarized in both directions (Yakir and Rahamimoff, 1995). Hence, under resting conditions, the channel is closed. However, it is known that after opening of the fusion pore (either transient or irreversible), the granule membrane potential rapidly collapses to that of the cell membrane (Breckenridge and Almers, 1987a). If vesicle fusion occurs in the vicinity of the peak of the action potential, then the vesicle membrane will be depolarized, near 0 mV, and the nonspecific channel will open, leading to the entry of cations into the vesicle and to facilitation in transmitter release. In the case of a negatively charged gel matrix and during the peak of an action potential, the ion channels can pass mainly cations into the lumen of the gel, since the majority of the anions contained in the gel matrix are fixed and unable to move. The ionic current flowing through the ion channels must always flow through the granule matrix and the fusion pore. When a cationic current crosses the ion exchange gel, the charge carrier of this current (e.g., K^+) will effectively displace the cationic secretory products that were initially present, forcing their release through the fusion pore. The longer the duration of the action potential, the longer the nonspecific channel will be in the open state, facilitating the release of secretory products by ion exchange. After the return of the membrane potential of the nerve terminal to the resting level, the nonspecific ion channel will close, even if the fusion pore is still open. Thus, the duration of the action potential may affect the efficiency of the release process through the fusion pore. Hence, the properties of th action potential may also have a postfusion role in the rate of transmitter release.

An obvious question is what is the relative contribution of the fusion pore and the vesicle channels in the supply of cations to the vesicle interior during the release process? This relative contribution will be determined by the conductances of the vesicle channels and fusion pore, by the number of open channels, by the driving forces, and by the relative affinity of the different cations for the ion exchange matrix. We have at present only partial answers to these important questions.

Caveats and Conclusions

Regulation of release after fusion is an attractive idea; however, it has to be considered with caution. The only cell where these ideas can be tested directly at the present time is the mast cell, where it is possible to record simultaneously the fusion of a single secretory granule and the resulting release of secretory products (Alvarez de Toledo et al., 1993). Although the presence of ion exchange gels has been firmly established in mast cell granules, it remains unclear whether these granules possess active ion channels. Hirashima and Kirino (1992) reported the presence of a cation selective ion

channel in the membrane of mast cell granules. However, we failed to observe these channels in isolated patch-clamped mast cell granules (Oberhauser and Fernandez, 1993). Furthermore, there is no published evidence that the release of serotonin during mast cell secretion is voltage dependent. It is possible that in mast cells, intracellular counterion fluxes use a mechanism that differs from that of ion channels such as active ATPases in the granule membrane. It is also possible that mast cells do not depend on intracellular counterion fluxes for their secretory activity and that all the counterions are supplied through the open fusion pore. However, mast cells are not excitable cells and do not require rapid mechanisms of secretion. In the rapid events that occur during synaptic transmission, it is likely that intracellular sources of counterions become more important.

The postfusion hypothesis has several obvious constraints when applied to synaptic transmission. If most of the transmitter is released from the synaptic vesicle as a "catastrophic" complete rapid fusion of the synaptic vesicle with the nerve terminal membrane, then the entry of ions through the vesicular channel has a limited importance. The intravesicular ion exchange matrix will face, under such circumstances, the extracellular fluid. Then the exchange of the transmitter with the cations in the extracellular space will determine the release process. On the other hand, if a substantial fraction of the transmitter quanta are released by the transient fusion mechanism, then the entry of counterions through a vesicular channel can be of considerable importance.

In this review, we presented the hypothesis that the release of substances from secretory granules and synaptic vesicles may be regulated after the onset of vesicle fusion by the size of the fusion pore, by the diffusivity of a neurotransmitter within its storage matrix, and by the source and availability of counterions during release by ion exchange. The consequences of these regulatory mechanisms for synaptic transmission are not yet entirely clear. However, as a broad generalization, one can visualize the synaptic delay as a representation of the prefusion control of transmitter release. The postfusion control will be reflected in the size and shape of the unitary quantal events. Future experiments will have to examine whether the proposed novel forms of regulating transmitter release discussed in this review are relevant for synaptic transmission.

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